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Application of multicolor flow cytometry in liquid biopsy of breast cancer

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ABSTRACT

As a result of the clinical study NCT04817501 “Phenotypic characterization of circulating tumor cells (CTCs) in tumors of the female reproductive system”, we developed a method for preoperative prediction of a recurrence risk in patients with stage T1 endometrial cancer (Patent No. 2762493 of 21.12.2021).

The article presents a clinical case of the use of multicolor flow cytometry in liquid biopsy of breast cancer (BC). CTCs were detected in the blood of a patient with T2N0M0 BC, stage IIA before the initiation of treatment. Using multicolor flow cytometry, various CTC phenotypes were studied and the Her2/neu and ki-67 markers were determined. These markers were also studied in the biopsy and surgical material of the BC tissue using immunohistochemistry. As a result of the study, it was shown that the molecular profile of CTCs in the blood taken before fine needle aspiration biopsy coincided with that of cancer cells in the BC tissue. In addition, the calculated risk of tumor progression before biopsy predicted recurrence of cancer in this patient 20 months before its occurrence. The obtained results show the practical utility of multicolor flow cytometry in liquid biopsy of cancers. The ability to evaluate CTCs by various molecular parameters can be useful for diagnosing, predicting, monitoring, and determining treatment strategies for cancer patients.

Keywords: liquid biopsy, breast cancer, multicolor flow cytometry, Her2/neu, ki-67, circulating tumor cells

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Применение многоцветной проточной цитометрии в жидкостной биопсии рака молочной железы

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РЕЗЮМЕ

В результате проведения клинического исследования NCT04817501 «Фенотипический спектр циркулирующих опухолевых клеток (ЦОК) при опухолях женской репродуктивной системы» разработан способ дооперационного прогнозирования риска рецидива у больных раком эндометрия Т1 стадии (патент № 2762493 от 21.12.2021).

Представлен клинический пример применения многоцветной проточной цитометрии в жидкостной биопсии рака молочной железы (РМЖ). В крови больной РМЖ T2N0M0 IIA стадии до начала лечения были выявлены ЦОК. Методом многоцветной проточной цитометрии исследованы различные фенотипы ЦОК и определены маркеры Her2/neu и Ki-67. В биопсийном и операционном материале ткани РМЖ методом иммуногистохимии были также исследованы данные маркеры. В результате показано, что молекулярный профиль ЦОК в крови, взятой до процедуры тонкоигольной биопсии, совпадал с молекулярным профилем опухолевых клеток ткани РМЖ. Кроме этого, рассчитанный риск развития опухолевой прогрессии до биопсии спрогнозировал возникновение рецидива у данной пациентки за 20 мес до его появления. Полученные результаты показывают практическую пользу многоцветной проточной цитометрии в жидкостной биопсии онкологических заболеваний. Возможность оценки ЦОК по различным молекулярным параметрам может быть полезной для диагностики, прогноза, мониторинга и определения стратегии лечения больных раком.

Ключевые слова: жидкостная биопсия, рак молочной железы, многоцветная проточная цитометрия, Her2/neu, Ki-67, циркулирующие опухолевые клетки

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Breast cancer (BC) occupies a leading place in terms of incidence and is among the five deadliest cancers in the world and in Russia [1]. It is a well-known fact that the main reasons for failure to treat BC are recurrence of the disease and its hematogenous metastasis.

Circulating tumor cells (CTCs) are involved in both recurrence and metastasis of cancer. The CTC population is known to be heterogeneous [2–5]. The presence of CTCs is not always accompanied by metastasis formation, apparently because not all cancer cells that have entered the circulation have properties sufficient for it [2, 6]. Even localized tumors without clinically visible metastases have been shown to be sources of CTCs [3, 7, 8]. It is estimated that 3.2×10^6 tumor cells detach from one gram of tumor tissue per day, but most of them quickly proceed to apoptosis due to the loss of adhesion to the extracellular matrix, hemodynamic shear forces, or immune system attacks [9, 10].

Liquid biopsy was introduced as a new diagnostic concept in 2010 to analyze CTCs in the blood of cancer

patients and has now expanded to analyze circulating tumor-derived factors, in particular circulating tumor DNA (ctDNA), as well as extracellular vesicles (EVs), microRNAs, mRNAs, long non-coding RNAs, circulating extracellular proteins, and tumor-educated platelets (TEPs) [11–25].

Tomsk NRMC in collaboration with Tomsk Regional Cancer Dispensary carried out a clinical study NCT04817501 "Phenotypic characterization of circulating tumor cells (CTCs) in tumors of the female reproductive system", following which a method for preoperative prediction of a recurrence risk in patients with stage T1 endometrial cancer was developed (Patent No. 2762493 of 21.12.2021) [26]. The predictive model was based on multicolor flow cytometry data on the number of different CTC populations, including circulating cancer stem cells, CTCs with markers of epithelial – mesenchymal transition (EMT), and atypical / hybrid cell populations.

The aim of the study was to show the practical application of multicolor flow cytometry and the efficiency of the developed predictive model in the diagnosis and prognosis of breast cancer.

CLINICAL CASE

In March 2021, patient P, 48 years old, turned to an oncologist with complaints of a neoplasm in the left mammary gland. The examination by a mammologist revealed that the mammary glands were symmetrical; the nipples and halos were without abnormalities; abundant serous discharge from the nipple was observed; a tumor (4 cm) in the upper inner quadrant of the left mammary gland was detected by palpation.

No skin flattening was noted. Regional lymph nodes were not enlarged. The patient was referred for mammography, bone scintigraphy, and computed tomography (CT) of the chest. In addition, venous blood was taken from the patient to study the presence and molecular profile of CTCs using multicolor flow cytometry.

In the course of the study, using fluorochrome-conjugated monoclonal antibodies to CD45, Epcam, CK, muc16, CD44, CD24, CD133, Ncadherin, Her2/neu, Ki67, and NucBlu Live reagent, various populations of CTCs were identified. Liquid biopsy showed that 72% of CTCs were Her2/neu positive and 35% were Ki-67 positive (Fig. 1).

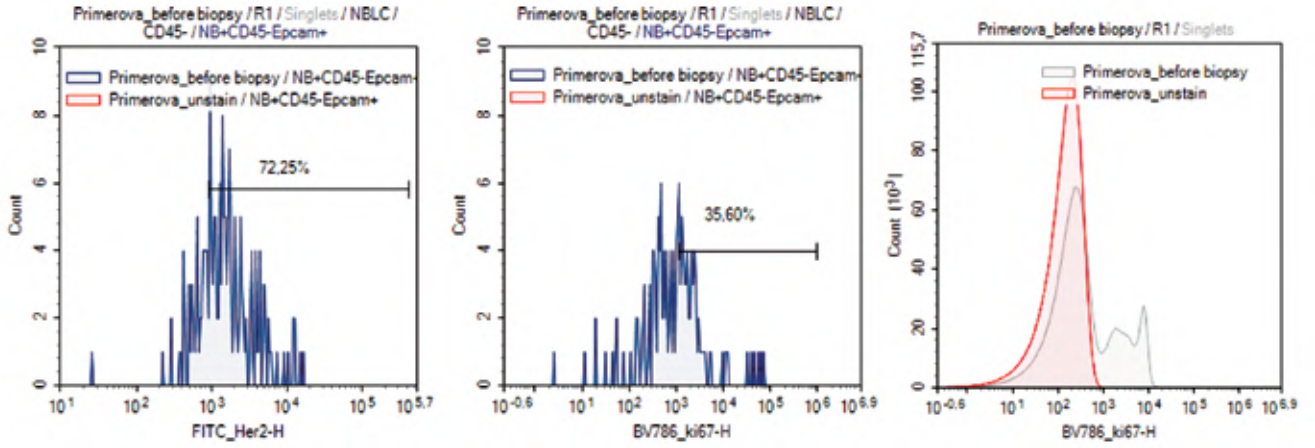

Fig. 1. Results of flow cytometry on the assessment of various populations of CTCs in the blood of patient P., 48 years old, before the biopsy

In addition, atypical / hybrid Epcam+CD45+ cells at a concentration of 19.3 cells / mm³ were detected in the blood of this patient. We calculated the risk of tumor progression using the model proposed in patent No. 2762493, based on data on the number of different CTC populations (the number of Epcam+CD45- cells, CTCs with the Epcam+CD45-CD44-CD24-Ncadherin+ phenotype, the number of circulating cancer stem cells without Epcam expression on the membrane with the Epcam(m)-CD45-CD44+CD24- phenotype (cells / ml), the number of atypical / hybrid forms of CTCs with the Epcam+CD45+ phenotype (cells / ml)). The risk of recurrence in this patient was 75%.

Mammography on the MG Adani machine (radiation exposure was 0.003 mSv) revealed accumulation

of pathological microcalcifications at the border of the inner quadrants of the LV. The area of changes was 44 × 19 × 26 mm. Impression: cancer of the left mammary gland manifested by microcalcifications. BI-RADS 5.

Chest CT of 31.03.2021 (CT Simens device, dose 4.80 mGy) revealed a mass in the left breast. No focal pathological lesions were found in the lungs.

Bone scintigraphy of 5.04.2021 using the pirfotechum radiopharmaceutical with an activity of 370 mBq and an effective radiation dose of 0.80 mSv did not reveal any scintigraphy signs of a focal skeletal lesion.

According to the ultrasound findings on the US Philips IU22 apparatus, a mass in the left mammary gland (34 × 11 × 17 mm) was found (BI-RADS 5). Axillary lymphadenopathy on the left was detected. Signs of diffuse fibrocystic breast disease were noted. The echotexture of the liver was not disturbed. Biopsy of the mass using a biopsy gun was performed. Impression of the histologic examination of the biopsy material (No. 8469-71\21) of 06.04.2021: non-specific invasive breast carcinoma (ICD-O code 8500/3), G 2 (3 + 2 + 1), with structures of ductal carcinoma *in situ*, G 2.

Immunohistochemistry of the biopsy material using the Leica Bond-Max immunohistochemistry staining system and antibodies against estrogen receptor (clone 6F11, Leica), progesterone receptor (clone 16, Leica), c-erB-2 (Her2/neu) (Polyclonal Rabbit, Dako), and Ki-67 (clone SP6, Cell Marque) showed positive homogeneous staining of tumor cells for estrogen receptors and heterogeneous staining for progesterone receptors (Fig. 2, *a, b*), Her2/neu 3+ membrane staining (Fig. 2, *c*); about 30% tumor cells were positive for the Ki-67 marker (Fig. 2, *d*), which corresponded to luminal B2 BC.

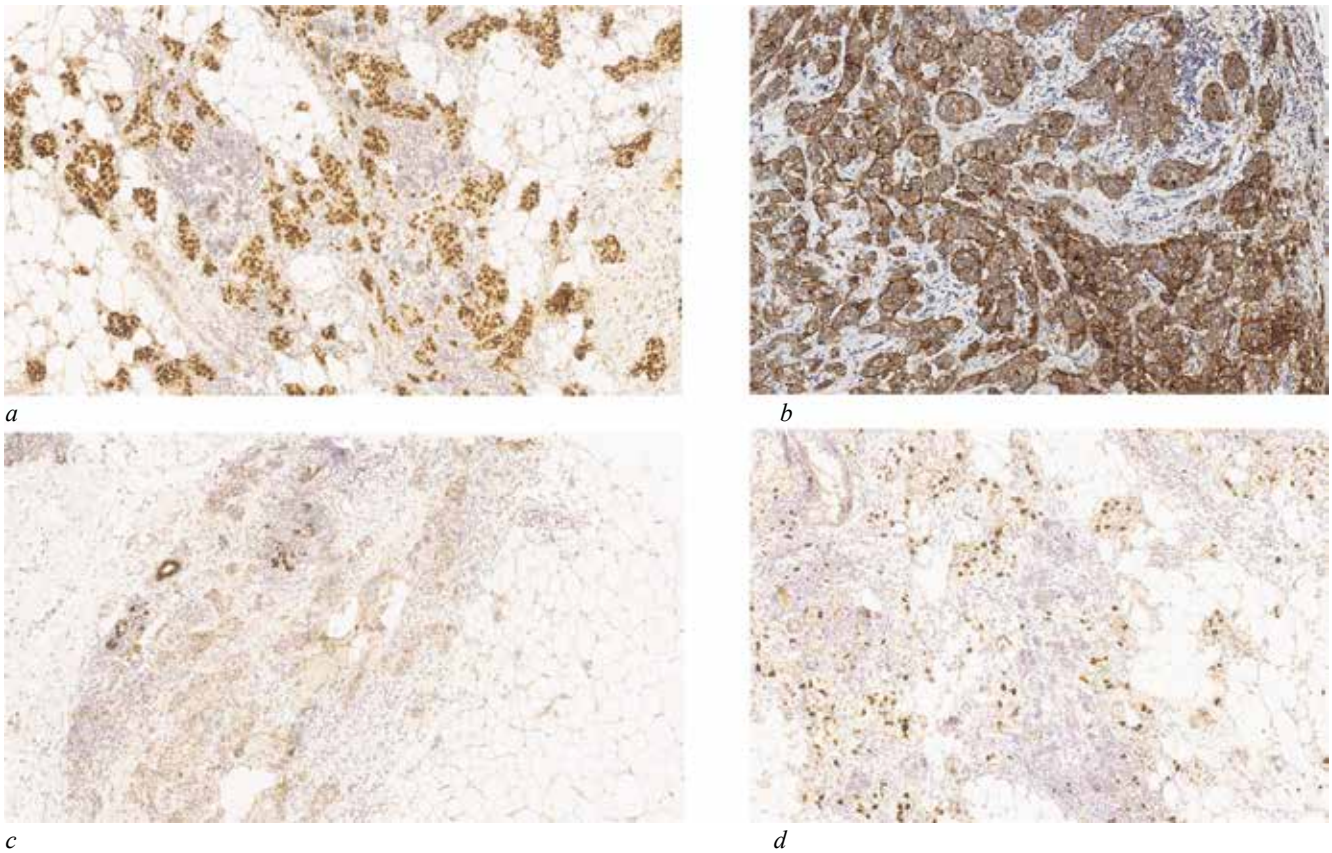

Fig. 2. Photographs of the immunohistochemical study of the biopsy material obtained from patient P., 48 years old. Immunohistochemical staining of breast cancer tissue using antibodies: *a* – against Estrogen receptor (clone 6F11, Leica), the image obtained using the digital slide scanner Aperio AT2, Leica at medium magnification; *b* – against progesterone receptor (clone 16, Leica); the image obtained using the digital slide scanner Aperio AT2, Leica at medium magnification; *c* – against c-erB-2 (Her2/neu) (Polyclonal Rabbit, Dako); the image obtained using the digital slide scanner Aperio AT2, Leica at high magnification; *d* – against Ki-67 (clone SP6, Cell Marque), the image obtained using the digital slide scanner Aperio AT2, Leica at medium magnification.

According to the results of the study, patient P., 48 years old, was diagnosed with cancer of the upper inner quadrant of the left breast (ICD code C50.2) T2N0M0, stage IIA. This patient underwent 6 courses of neoadjuvant chemotherapy (DCHP). In September 2021, she underwent a subcutaneous mastectomy on the left with a biopsy of the sentinel lymph nodes at Cancer Research Institute of Tomsk NRMC. Impression of sentinel lymph node cytology (slide no.: 2124cito 28.09.2021) showed the presence of lymphoid elements. The impression of the pathomorphological examination of the surgical material (No. 27486-5051): invasive carcinoma of no special type G2 (ICD-O code 8500/3). No metastatic lesion was detected in the sentinel lymph node. Curative pathomorphosis according to the RCB grading system – RCB-I, according to G.A. Lavnikova – III degree, pathological type ypT1N0Mx. No tumor was detected along the resection margins.

Until May 2022, the patient received transtuzumab, then tamoxifen. On 15.07.2022, according to the chest CT, areas of compaction in the lungs without dynamics were identified. The impression of the ultrasound examination of 15.07.2022: incomplete involution of the right breast. Residual signs of fibrocystic breast disease on the right. Condition after a subcutaneous mastectomy on the left, sentinel lymph node biopsy, expander installation (September 2021). No echoscopy evidence of disease progression was obtained. Diffuse changes in the liver were noted. Chronic cholecystitis. Bone scintigraphy of 21.11.2022 did not reveal any scintigraphy signs of non-proliferative focal skeletal lesions. The impression of the ultrasound examination of 18.11.2022: axillary lymphadenopathy on the left. Fine-needle aspiration biopsy was performed. The cytological examination of the lymph node puncture showed the presence of metastasis.

Thus, according to the results of the examination 13 months after the surgery, data for regional BC recurrence were obtained. In December 2022, the patient underwent axillary lymphadenectomy. The impression of the pathomorphological study (No. 39063-76/22 of 26.12.2022): metastasis of invasive breast carcinoma (ICD-O code 8500/6) to the lymph node, with invasion of the tumor into the capsule of the lymph node and extracapsular spread into the perinodal adipose tissue, signs of lymphovascular invasions. Data for neural invasion were not found. Currently, the patient has independently sought care at Blokhin National Medical Research Center of Oncology, where she is receiving radiation therapy and chemotherapy with anastrozole and transtuzumab.

CONCLUSION

Thus, the use of multicolor flow cytometry in liquid biopsy of BC made it possible to identify heterogeneous populations of CTCs. As a result of the study, it was shown that the molecular profile of CTCs in the blood taken before fine needle aspiration biopsy coincided with the molecular profile of tumor cells in the BC tissue. In addition, our calculated risk of tumor progression before biopsy predicted a recurrence of the disease in this patient 20 months before its occurrence. The obtained results show the practical utility of multicolor flow cytometry in liquid biopsy of cancers. The ability to evaluate CTCs by various molecular parameters can be useful for diagnosing, prognosing, monitoring, and determining a treatment strategy for cancer patients.

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